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The effects of lithium chloride on seed germination, seedling growth and antioxidant response in *Brassica oleracea* var. *capitata*

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ABSTRACT:
The widespread use of lithium (Li) in various industries, the low recycling rate of products with high Li content, and Li extraction and ore processing have resulted in Li becoming a contaminant of emerging concern with biological and ecological effects. Despite increased research over the past two decades, its influence on plants remains largely unknown and sometimes unclear. This study analyses the effects of elevated Li concentrations in the substrate (0–1000 mg kg^{-1}), added as LiCl, on *Brassica oleracea* var. *capitata* (cabbage) plants. We monitored the seed germination, seedling growth, nutrient status, photosynthetic pigment content, and antioxidant enzyme activities in plants grown hydroponically during two-week experiments. Lithium exhibited hormesis in some of the parameters analysed. The germination rate remained high (>80%) at all Li concentrations. In the earliest stages of plant growth, the lowest applied Li concentrations (25–100 mg L^{-1}) stimulated radicle elongation and root hair development, while higher concentrations caused their regression and led to premature seedling death. In the following two weeks, the Li-exposed plants showed a dose-dependent decrease in growth parameters and biomass yield. Lithium accumulated mainly in the leaves and affected the accumulation and translocation of various mineral elements in the plants, disrupting their content and stoichiometric ratios. In the leaves, all the applied Li concentrations caused a decline in photosynthetic pigment content. They also led to significant changes in antioxidant enzyme activities: superoxide dismutase (SOD), catalase, and class III peroxidases (POD) in the leaves, and SOD, ascorbate peroxidase, and POD activities in the roots, indicating the increased formation of reactive oxygen species. The overall results indicate that, despite the initial stimulatory influence, even low doses of Li exert inhibitory effects on plant growth and the examined physiological parameters in the subsequent period. Therefore, longer-term investigations are required, covering a broader range of growth, development, and functional parameters prior to plant maturation. This is particularly important given that cabbage is an economically important plant and one of the most commonly consumed vegetables in Serbia and across Europe.

Keywords: cabbage, enzyme, metal antagonism, metal tolerance, oxidative stress

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INTRODUCTION

The growing global demand for lithium (Li) is primarily driven by its essential role in manufacturing Li-based products, including lithium-ion and solid-state batteries used in portable electronics, electric vehicles, medical devices, and environmental sensors. Lithium is also used in ceramics, glass, catalysts, lubricants, and rocket fuels, often with significant processing losses (Fasel & Tran 2005; Scrosati & Garche 2010; Bonino *et al.* 2011). Future advancements in deuterium-tritium nuclear fusion are also likely to further increase Li demand (Fasel & Tran 2005). Currently, Li is mainly sourced from brine deposits, but production volumes remain insufficient to meet rising demand (Fasel & Tran

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2005). As a result, extraction from mineral reserves is on the increase despite its significant environmental impact—especially in mining regions (Al-Thyabat *et al*. 2013). Additional anthropogenic sources of Li pollution include improper battery disposal, emissions from Li-rich coal combustion, brine discharge, and waste from the recycling and incineration of electronics (Chalvatzaki *et al*. 2014).

Despite the undeniable global economic importance of Li for industrial applications, the rapid expansion of Li-related industries and lithium-containing products has raised considerable concern about environmental impacts, particularly the contamination of agricultural areas and water bodies (Shahzad *et al*. 2016; Bibienne *et al*. 2020). Already present at elevated concentrations in some surface waters and agricultural soils, Li has attracted the attention of both the general public and the scientific community and is now considered a serious new environmental pollutant. Geogenic Li occurs within structurally bound, insoluble mineral phases, and its bioavailability to plants is primarily governed by pedogenetic processes, including the rate of mineral chemical weathering and the soil's physicochemical characteristics. In contrast, Li released into the environment through anthropogenic activities is typically present in more soluble and mobile forms, resulting in its greater availability within the soil (Robinson *et al*. 2018; Bolan *et al*. 2021). Due to its high mobility, Li and its compounds can easily leach into surface water and groundwater, making them highly bioavailable with a tendency to bioaccumulate in certain biota (Robinson *et al*. 2018). The limited available data on Li suggest that it is readily absorbed by plant roots, while its ascending translocation to aerial parts depends on the Li concentration in the growth substrate, typically resulting in the highest accumulation in the roots (Kastori *et al*. 2022). However, different plant species show substantial variation in Li uptake and accumulation ability, even under identical environmental conditions. Thus, its concentrations in plants are found within a very broad range, with values between 0.15 and 6000 mg kg^{-1} (Scrosati & Garche 2010; Bonino *et al*. 2011; Kavanagh *et al*. 2018; Kastori *et al*. 2022). The highest values are found in plants belonging to the Asteraceae, Rosaceae, and Solanaceae families, as well as in halophilic plants (Schrauzer 2002; Kabata-Pendias & Mukherjee 2007). In spite of good absorption by plants, Li is not considered an essential or beneficial element for plants and its role in plant physiology remains the subject of debate (Shahzad *et al*. 2016; Kastori *et al*. 2022). Currently, there is no evidence that it functions as a cofactor in metalloenzymes or is involved in any other biological process in plants. Despite the very limited understanding of its biological roles, Li is known to replace essential elements such as Na^+, K^+, Ca^{2+}, and Mg^{2+} due to its small ionic radius and high polarisation strength (Zhong *et al*. 2015; Shahzad *et al*. 2017). Such Li-induced cation imbalance in the cells results in modified structure and the disrupted functionality of metalloproteins, membranes and other cell components to which it binds. Yet, as in the case with numerous other metals, Li manifests hormetic effects: at low concentrations it has been reported to stimulate plant growth and enhance disease resistance (Shahzad *et al*. 2016, 2017). The exact value of such low concentrations depends on plant species and genotype, differing, for instance, between more tolerant and Li-sensitive plants. At elevated concentrations Li shows dose-dependent inhibition of seedling growth and becomes toxic, particularly for plants with low Li tolerance. The meta-analysis shows that elevated concentrations of Li inhibit root and shoot growth, decrease chlorophyll *a* and *b* content in leaves, increase oxidative stress, induce changes in enzymatic and non-enzymatic antioxidant levels, and induce foliar damage such as localised necrotic lesions or leaf curling (Naranjo *et al*. 2003; Kastori *et al*. 2022; Shakoor *et al*. 2023; Kapoor & Hasanuzzaman 2024). Insights into the precise mechanisms underlying lithium's effects in the plant cell remain unclear, and further research is still required to determine

and understand the mechanisms of both its positive and negative influences on various metabolic processes.

Brassica oleracea var. *capitata* L. is economically important and among the most widely used edible plants in the human diet in Serbia and Europe. The objectives of this study were: (a) to examine the accumulation rate of Li in below- and above-ground plant parts of seedlings grown hydroponically in a short-term experiment; (b) to analyse the effects of accumulated Li on the uptake and distribution of essential mineral elements (K, Na, Ca, Mg, Zn, Cu, Mn) within the plant and their potential interactions; and (c) to evaluate the effects of elevated Li on seed germination, plant growth, photosynthetic pigment contents, and the activities of antioxidant enzymes in the roots and shoots.

MATERIALS AND METHODS

Seed germination. Dry seeds of cabbage (300 seeds per treatment) were surface sterilised with bleach solution (30% commercial bleach) for 1 minute and then washed well in distilled water. The seeds were then placed in Petri dishes directly on quantitative filter paper moistened with the Hoagland No. 2 basal salt mixture (Sigma Lot # SLBK2642V) added with different Li concentrations (25, 50, 100, 200, 400, 600 and 1000 mg Li L^{-1}). The petri dishes were incubated in the dark for 2 days, after which the number of germinated seeds was counted and expressed as a percentage. The 48-hour-old seedlings were observed using Leica a MZ 7_5 stereomicroscope and photographed using a Leica DFC 295 camera.

Cultivation of the plants with different concentrations of lithium. A few days after germination, the seedlings were transferred to plastic cups filled with 100 mL of the Hoagland No. 2 solution, supplemented with the above-listed Li concentrations in the form of LiCl, and the lethal concentrations were determined. A new set of plants was then cultivated hydroponically in Hoagland No. 2 Basal Salt Mixture containing different concentrations of Li: control (containing 18 µM Li, as the standard content in applied salt), Li_{25} (25 mg Li L^{-1} = 3.618 mM Li), Li_{50} (50 mg Li L^{-1} = 7.218 mM Li) and Li_{100} (100 mg Li L^{-1} = 14.418 mM Li). The pH of the nutrient solution was 6.43 ± 0.06 (control), 6.23 ± 0.06 (Li_{25}), 6.33 ± 0.12 (Li_{50}), 6.40 ± 0.20 (Li_{100}) (pH meter Testo 545). Each container held 66 positions filled with clay balls of 8–16 mm in diameter (Plagron, Euro Pebbles), previously rinsed with nutrient solution, on top of which a mesh with seeds was placed. The nutrient solution was aerated daily using an air pump (SB 108, Sebo). For 14 days, the plants were grown under controlled conditions in a plant growth chamber with a 16/8 hour photoperiod. The photosynthetic photon flux density (PAR) in different parts of the chamber ranged from 109 to 165 µmol PPFD $m^{-2}s^{-1}$, requiring multiple rotations of the containers during plant growth. The air temperature during the illuminated period ranged from 23.3 to 26.3°C. After 14 days, the plants were sampled and prepared for further analyses.

Elemental composition of the plants. Each seedling was separated into the roots, stems and cotyledons (with leaves), washed carefully in tap water and then in deionised water to remove any traces of the nutrient solution from their surface. The plant material was dried overnight at 70°C, then finely ground using a ceramic mortar and pestle. The powdered plant material was dried to a constant weight at 100°C, and subsequently digested in 65% HNO_3 at 150°C (Jones & Case 1990). The absorption/emission values of the elements (Li, Ca, Mg, K, Na, Cu, Mn, Zn and Fe) in the plant samples were obtained using an atomic absorption spectrophotometer (Shimadzu AA - 7000). The concentrations of the elements were determined by comparing their absorption/emission values with those of known standards (Jones & Case 1990). The suitability of the analytical procedure for the determination of the metal content was con-

firmed by using the standard reference material (NIST 1515 apple leaves). The limits of detection for Li, Ca, Mg, K, Na, Cu, Mn and Zn are 1.2, 17, 2.0, 98, 35, 0.7, 0.7 and 0.3 mg kg dw (dry weight)$^{-1}$, respectively.

Pigment quantification. The content of photosynthetic pigments in the leaves was detected according to Hiscox & Israelstam (1979) using dimethyl sulphoxide as the solvent. The absorbance of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and total carotenoids (Car) was determined by a UV–Vis spectrophotometer (Jenway 7315 Spectrophotometer) at wavelengths of 480 nm, 663 nm and 645 nm. The pigment concentrations were calculated using the equations given by Arnon (1949) and Wellburn (1994), and were expressed as mg g^{-1} leaf dry weight.

Analyses of the antioxidant enzyme activities. For protein extraction and the determination of protein content, the whole-cell protein extract used for the analysis of enzyme activities was obtained by pulverizing approximately 0.5 g of fresh plant material in liquid nitrogen and homogenizing it in 1 ml of extraction buffer (100 mM K-phosphate buffer (KPi) at pH 7, 5% polyvinylpyrrolidone and 0.1 mM EDTA). The homogenates were then centrifuged at 13000×g for 30 minutes at 4°C. The protein concentrations of the extracts were determined spectrophotometrically (Shimadzu UV-1800) according to the method proposed by Bradford (1976), using bovine serum albumin as the protein standard.

For the enzyme assays, the activity of superoxide dismutase (SOD) was determined indirectly by the nitro-blue tetrazolium test (NBT) (Beauchamp & Fridovich 1971). The reaction mixture contained 100 mM KPi buffer (pH 7), 0.1 mM EDTA, 13 mM methionine, 2 μM riboflavin, 75 μM NBT and 30 μL plant extract in 1 mL final volume. After incubating the samples in fluorescent light for 15 minutes, the absorbance of the photochemically reduced NBT was measured at 560 nm (Shimadzu UV-1800). The activity of catalase (CAT) was determined by measuring the H_2O_2 consumption at 240 nm (Aebi 1984). The reaction mixture contained 100 mM KPi buffer (pH 7), 0.1 mM EDTA, 20 mM H_2O_2 and 20 μL extract in 1mL final volume. The ascorbate peroxidase (APX) activity was determined by detecting ascorbic acid (AsA) oxidation and the decrease in absorbance was monitored at 290 nm over time (Nakano & Asada 1981). The reaction mixture contained 100 mM KPi buffer (pH 7), 0.1 mM EDTA, 0.5 mM AsA, 0.1 mM H_2O_2 and 30 μL of the plant extract in a final volume of 1 ml. The activity of peroxidase (POD) was determined by measuring the formation of tetraguaiacol (Polle *et al.* 1994). The reaction mixture consisted of 100 mM KPi buffer (pH 7), 0.1 mM EDTA, 9 mM guaiacol, 20 mM H_2O_2 and 30 μL extract in a final volume of 1 mL. The enzyme activities were determined spectrophotometrically at 25°C, and were expressed as U mg^{-1} of protein.

Plant biometric parameters. Twenty seedlings per treatment were used for plant dry weight determination. Furthermore, 12 plants per treatment were fixed in a 50% ethanol:glycerol mixture (1:1, v/v), scanned and used for the measurement of the biometric parameters: the total root length, the stem length and the cotyledon area.

Data analysis. Data were expressed as mean ± standard deviation (M ± SD) of five (for physiological parameters and elemental analyses) or more (12–20) replicates. The Mann-Whitney U test was used to determine any significant differences between the analysed groups. The correlations between variables were calculated using Spearman's rank correlation coefficient (ρ). Statistical analyses were performed using R software (v3.5.1; R Core Team 2018).

Table 1. The effects of different Li concentrations on germination percentages of *Brassica oleracea* var. *capitata* seeds. Different letters indicate significant differences among the treatments with the Mann-Whitney U test ($p < 0.05$)

Treatment	Control	Li_{25}	Li_{50}	Li_{100}	Li_{200}	Li_{400}	Li_{600}	Li_{1000}
Germination rate (%)	94.1 ± 1^{b}	94.7 ± 3.1^{ab}	94 ± 0^{b}	92 ± 1^{ab}	90.7 ± 2.3^{ab}	91.3 ± 1.2^{ab}	84.7 ± 3.1^{a}	82.7 ± 4.7^{a}

Table 2. Growth parameters in *Brassica oleracea* var. *capitata* grown at different Li concentrations. Different letters indicate significant differences among the treatments with the Mann-Whitney U test ($p < 0.05$)

Group	Total root length (mm)	Stem length (mm)	Cotyledon area (mm)	No. of leaves	Dry biomass shoot (mg)	Dry biomass root (mg)
Control	173 ± 37^{b}	81.7 ± 23^{c}	157 ± 35^{d}	2.9 ± 0.3^{b}	21 ± 3^{c}	2.5 ± 0.8^{b}
Li_{25}	170 ± 43^{b}	63.8 ± 9.9^{b}	70.3 ± 29^{b}	2.8 ± 0.4^{b}	11.1 ± 1.5^{b}	2.2 ± 0.4^{b}
Li_{50}	176 ± 41^{b}	63.7 ± 10.2^{b}	90.2 ± 25^{c}	3.2 ± 0.6^{b}	5.6 ± 0.5^{b}	0.6 ± 0.6^{a}
Li_{100}	11.3 ± 4.4^{a}	16.8 ± 12.9^{a}	18.6 ± 5.6^{a}	2 ± 0^{a}	2.2 ± 0.4^{a}	0.4 ± 0.3^{a}

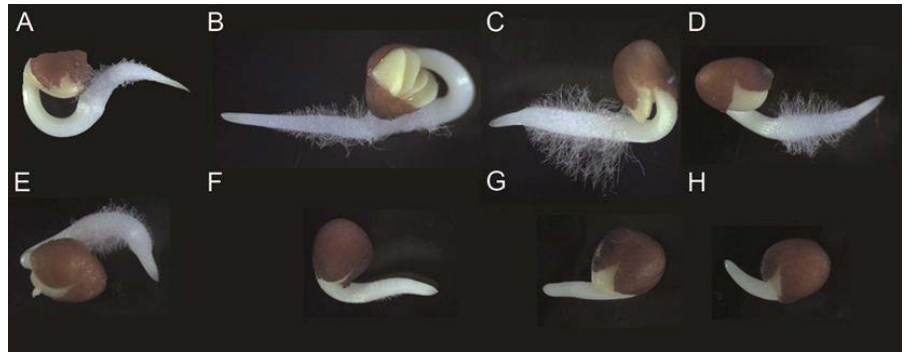

Fig. 1. Radicle and root hair development in *Brassica oleracea* var. *capitata* 48 h after germination; A-control, B-25 mg Li L^{-1}, C-50 mg Li L^{-1}, D-100 mg Li L^{-1}, E-200 mg Li L^{-1}, F-400 mg Li L^{-1}, G-600 mg Li L^{-1}, H- 1000 mg Li L^{-1}

The capacity for metal accumulation in the roots and cotyledons was assessed using the bioaccumulation factor (BAF) and bioconcentration factor (BCF) which represent the ratio between the metal concentration in the roots and the substrate and between the metal concentration in the cotyledons and the substrate, respectively. The efficiency of metal ascending translocation within the plant was assessed using the translocation factor (TF), which represents the ratio between the metal concentration in the cotyledons and the roots.

RESULTS

Seed germination. After 48 hours of incubation no toxic effects of low Li concentrations (25–50 mg Li L^{-1}) were detected in the *B. oleracea* var. *capitata* seeds, and their germination rates were comparable to the control (Table 1). Although moderate concentrations of applied Li (100–400 mg L^{-1}) slightly reduced the seed germination rate, the seeds exposed to the highest applied Li concentrations (600 and 1000 mg L^{-1}) retained high germination rates (>80%), which were only 12% lower than the control.

Effects of lithium on plant growth. The two-day old seedlings show that the development of the primary root and root hairs was stimulated by lower Li concentrations (25–100 mg Li L^{-1}) (Fig. 1), while in the seedlings exposed to higher lithium concentrations this noticeably slowed (400–1000 mg Li L^{-1}).

The two-week old seedlings showed a dose-dependent reduction in shoot and root dry biomass, stem length, and cotyledon area (Table 2). Although all the Li-treated plants exhibited a significant decline in overall growth, this effect was particularly pronounced in the Li_{100} plants, which also displayed necrotic lesions along the cotyledon and leaf margins. Additionally, the Li_{100} plants also exhibited delayed first leaf development (by 30%) compared to the other Li-treated plants (Li_{25}, Li_{100}) (data not shown). All the other plants exposed to Li concentrations above 100 mg L^{-1} died within a few days of germination.

Metal concentrations in the plants. The concentration of Li in the roots and stems of the Li-treated plants increased in parallel with the Li concentration in the hydroponic solution (Fig. 2). The lithium concentration in the leaves was several-times higher than that detected in the roots and stems, with the highest value observed in the Li_{50} plants. The calculated BAFs for Li in the plants were 88 (control), 10.5 (Li_{25}), 9.7 (Li_{50}) and 9.3 (Li_{100}), the BCF values were 41 (control), 123 (Li_{25}), 181 (Li_{50}) and 69 (Li_{100}), while the TFs were 0.5 (control), 11.7 (Li_{25}), 18.7 (Li_{50}) and 7.4 (Li_{100}). In contrast to the distribution pattern of Li, divalent ions such as Ca, Cu, Mn and Zn were predominantly accumulated in the plant roots.

In the roots, the effects of Li on cation absorption were indicated by strong positive correlations ($p < 0.001$) between Li and concentrations of Cu ($\rho = 0.956$), Mn ($\rho = 0.937$), Zn ($\rho = 0.872$) and K ($\rho = 0.666$, $p<0.01$) (Table 3). In contrast, in the cabbage leaves predominant lithium accumulation was strongly positively correlated with concentrations of Mn ($\rho = 0.753$, $p<0.001$) and Zn ($\rho = 0.666$, $p < 0.01$) (Table 4).

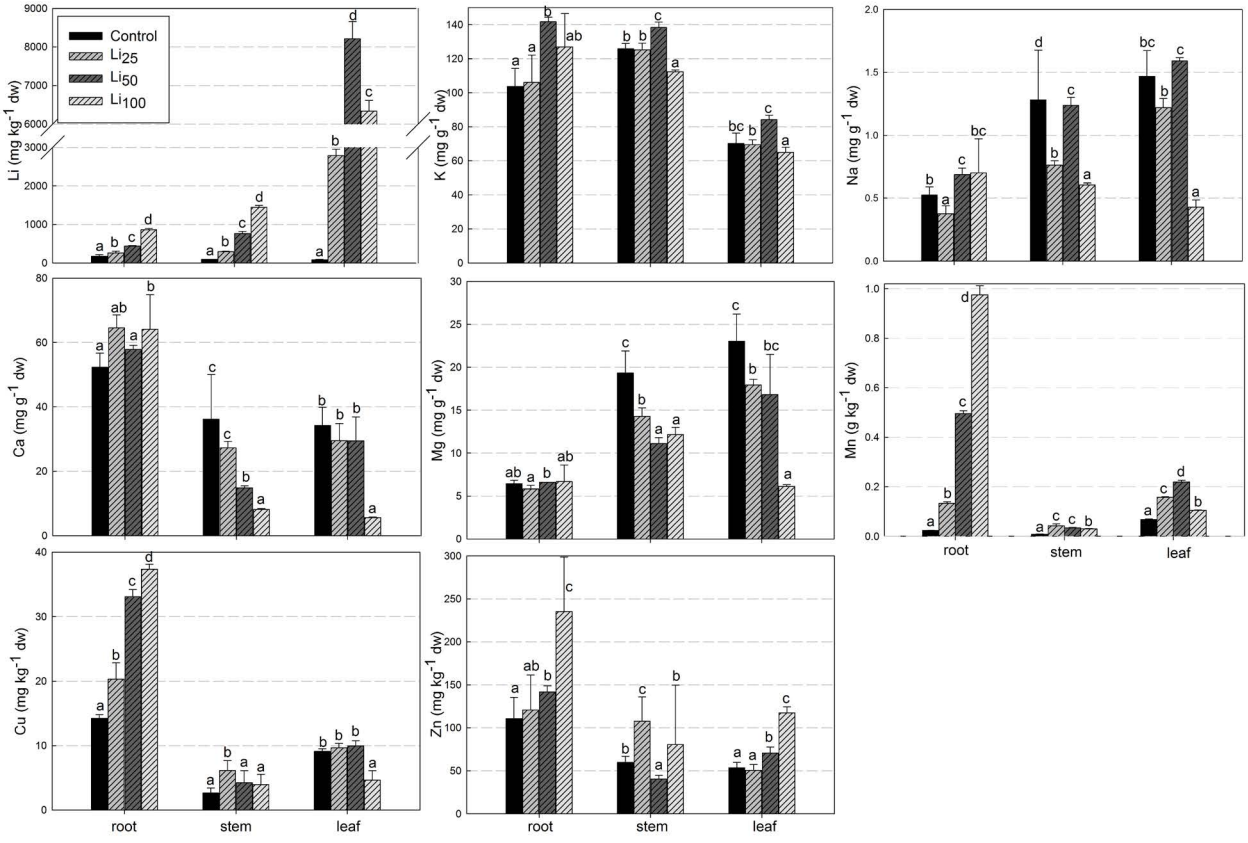

Fig. 2. The metal content of the roots and leaves of *Brassica oleracea* var. *capitata* seedlings grown under elevated Li concentrations. Different letters indicate significant differences among the treatments with the Man-Whitney U test ($p < 0.05$)

Table 3. Spearman's rank order correlation coefficients for the mineral element concentrations and antioxidant enzyme activities in the roots of *Brassica oleracea* var. *capitata* grown at different Li concentrations (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

	Li	K	Na	Ca	Mg	Cu	Mn	Zn	SOD	CAT	APX
Li	1.000										
K	0.666**	1.000									
Na	0.657**	0.665**	1.000								
Ca	0.547*	0.235	0.087	1.000							
Mg	0.053	0.347	0.556**	-0.197	1.000						
Cu	0.956***	0.642**	0.671**	0.546*	0.131	1.000					
Mn	0.937***	0.621**	0.693***	0.555*	0.191	0.970***	1,000				
Zn	0.872***	0.662**	0.684***	0.495*	0.260	0.916***	0.901***	1.000			
SOD	0.334	-0.161	0.337	0.093	0.123	0.347	0.323	0.314	1.000		
CAT	0.045	-0.257	-0.200	0.265	-0.120	-0.027	-0.008	-0.021	0.176	1.000	
APX	-0.865***	-0.710***	-0.558*	-0.370	-0.024	-0.875***	-0.809***	-0.771***	-0.060	0.144	1.000
POD	0.617**	0.131	0.384	0.375	-0.154	0.557*	0.577**	0.436	0.438	0.351	-0.378

Table 4. Spearman's rank order correlation coefficients for the mineral element concentrations, antioxidant enzyme activities, chlorophyll *a* and *b*, and total carotenoids in the leaves of *Brassica oleracea* var. *capitata* grown at different Li concentrations (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

	Li	K	Na	Ca	Mg	Cu	Mn	Zn	SOD	CAT	APX	POD	Chl *a*	Chl *b*
Li	1.000													
K	0.430	1.000												
Na	-0.005	0.479*	1.000											
Ca	-0.260	0.421	0.521*	1.000										
Mg	-0.519*	0.272	0.668**	0.743***	1.000	0.405								
Cu	0.132	0.580*	0.597**	0.615**	0.405	1.000								
Mn	0.753***	0.656*	0.180	0.103	-0.276	0.591**	1.000							
Zn	0.666**	-0.011	-0.394	-0.575**	-0.603*	-0.471*	0.151	1.000						
SOD	0.395	-0.138	-0.239	-0.562**	-0.474**	-0.518*	-0.086	0.476*	1.000					
CAT	0.707***	0.308	-0.059	-0.197	-0.508*	0.152	0.682***	0.462*	0.122	1.000				
APX	0.155	0.298	0.597**	0.132	0.131	0.377	0.204	-0.192	0.152	-0.075	1.000			
POD	0.081	-0.320	-0.511*	-0.456*	-0.433	-0.708***	-0.362	0.603**	0.266	0.069	-0.316	1.000		
Chl *a*	-0.591**	-0.014	0.490*	0.497*	0.668**	0.356	-0.231	-0.641**	-0.668**	-0.236	-0.060	-0.493*	1.000	
Chl *b*	-0.650**	0.017	0.515*	0.593**	0.779***	0.411	-0.269	-0.714***	-0.729***	-0.337	-0.050	-0.483*	0.965***	1.000
Tot Car	-0.531*	-0.030	0.451*	0.327	0.699***	0.177	-0.393	-0.412	-0.515	-0.253	-0.164	-0.197	0.815***	0.829***

The antagonistic or synergistic effects of Li in the leaves were most pronounced at the highest applied Li concentration (100 mg L^{-1}), significantly reducing the contents of Na, Ca, Mg and Cu, and increasing Zn and Mn concentrations. The potential effects of accumulated Li on plant metabolism can be assessed by using both changes in metal concentrations and those in metal molar ratios (Table 5). The element stoichiometry in the roots, stems, and leaves of *B. oleracea* var. *capitata* seedlings grown under elevated Li content is shown in Table 5.

Table 5. Element stoichiometry in the roots, stem and leaves of *Brassica oleracea* var. *capitata* seedlings grown under elevated Li content

		K:Li	Na:Li	Ca:Li	Mg:Li	Li:Zn	Li:Cu	Li:Mn
roots	C	106	0.9	53	11	15	111	59
	Li_{25}	73	0.4	44	6.6	20	115	15
	Li_{50}	52	0.4	21	3.9	32	134	7.8
	Li_{100}	24	0.2	12	2.0	37	228	7.5
stem	C	240	4.1	54	60	15	323	89
	Li_{25}	78	0.8	17	14	25	425	53
	Li_{50}	29	0.4	3.1	3.8	196	1811	194
	Li_{100}	13	0.1	0.9	2.3	186	3706	421
leaf	C	149	5.3	71	79	15	84	9.8
	Li_{25}	4.0	0.1	1.7	1.7	575	2918	154
	Li_{50}	1.7	0.1	0.6	0.5	1206	8312	327
	Li_{100}	1.7	0.0	0.1	0.3	550	13651	515

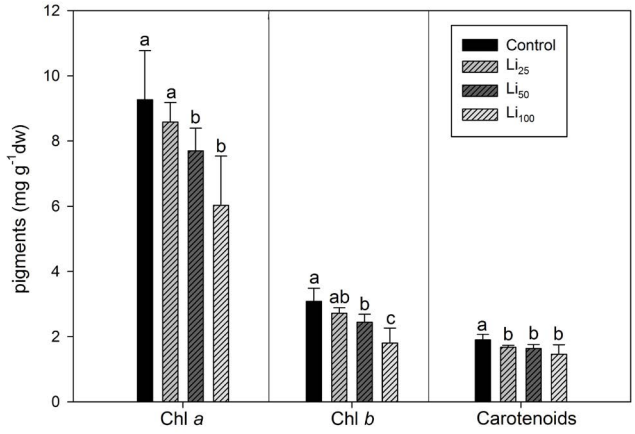

Fig. 3. The photosynthetic pigment content in the leaves of *Brassica oleracea* var. *capitata* seedlings grown under elevated Li content. Different letters indicate statistically significant differences among the treatments (Man-Whitney U test, $p < 0.05$)

Effects of lithium on the photosynthetic pigments and antioxidant enzymes. An increase in Li concentration in the leaves was accompanied by a decrease in the concentration of photosynthetic pigments with Chl *a*, Chl *b* and carotenoids being 35%, 41%, and 36% lower in the Li_{100} plants compared to the control (Fig. 3).

Antioxidant enzymes, including SOD and POD, significantly increased in both the roots and leaves only in those plants grown under the highest applied Li concentration (Li_{100}) (Fig. 4). In the roots, there was no significant change in CAT activity, whereas in the leaves it was twofold higher in all the Li-treated plants compared to the control plants. APX activity was reduced in the roots of all the Li-treated plants, as well as in the leaves of the Li_{100} plants. Leaf SOD activity showed negative correlations with Ca (ρ = -0.562), Mg (ρ = -0.474), Cu (ρ = -0.518), and a positive correlation with Zn (ρ = 0.476) (Tables 4 & 5). Catalase activity in the leaves showed statistically significant positive correlations with Li (ρ = 0.707), Mn content (ρ = 0.682) and Zn (ρ = 0.462), and a negative correlation with Mg (ρ = -0.508). In the roots, POD activity showed positive correlations with Li (ρ = 0.617), Cu (ρ = 0.557), and Mn (ρ = 0.577), whereas in the leaves it showed a positive correlation with Zn (ρ = 0.603) and a negative correlation with Ca (ρ = -0.456). Ascorbate peroxidase in the roots showed strong negative correlations with Li (ρ = -0.865), K (ρ = -0.710), Cu (ρ = -0.875), Mn (ρ = -0.809) and Zn (ρ = -0.771), while in the leaves it showed a positive correlation with Na (ρ = 0.597).

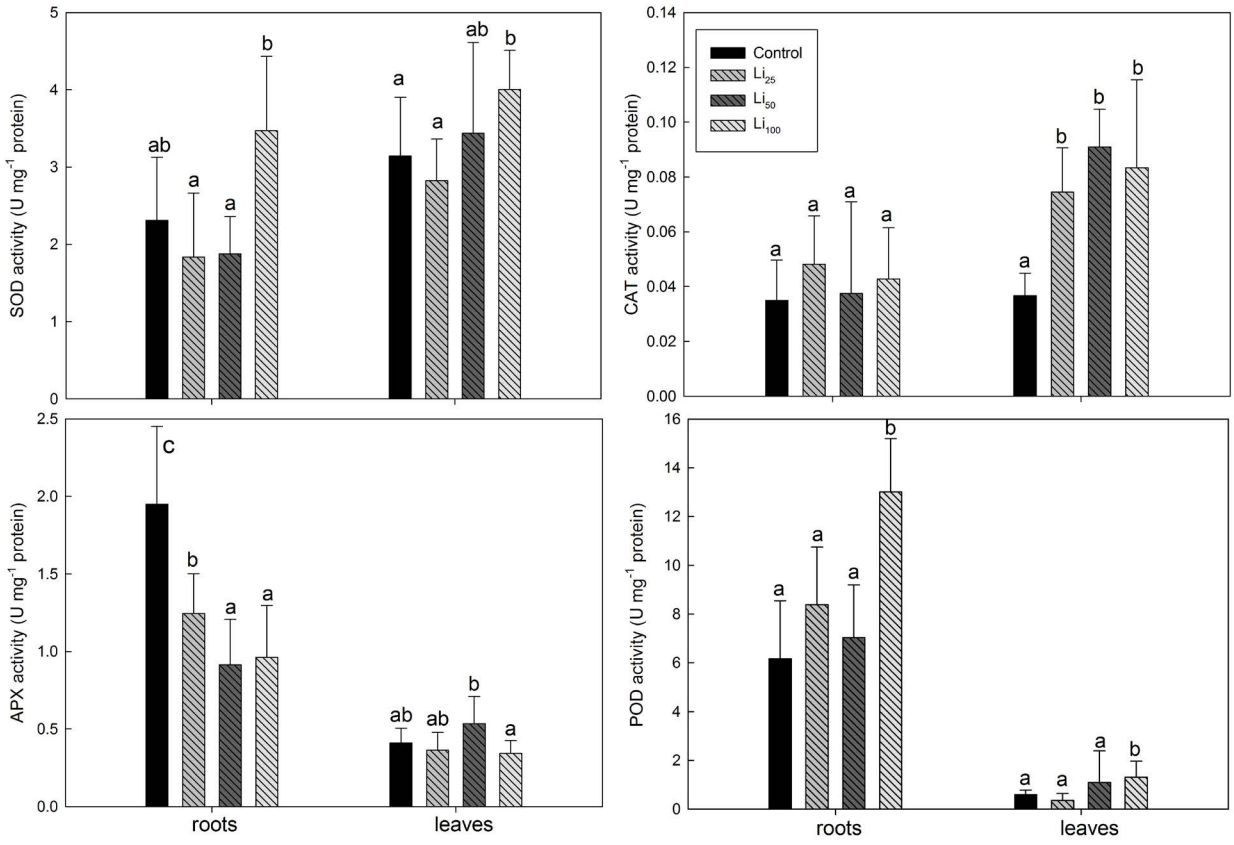

Fig. 4. Antioxidant enzyme activities in the roots and leaves of *Brassica oleracea* var. *capitata* seedlings grown under elevated Li content. Different letters indicate significant differences among the treatments with the Man-Whitney U test ($p < 0.05$)

DISCUSSION

Effects of elevated Li content on seed germination and seedling growth. By achieving high germination rates, even at the highest applied Li concentrations, the *Brassica oleracea* var. *capitata* seeds demonstrated very good initial Li tolerance. Inhibitory effects on seed germination were not observed at low to medium Li concentrations (Li $\leq$ 400 mg L^{-1}) and occurred only at the highest concentrations applied (600 and 1000 mg L^{-1}), with the germination rate still exceeding 80%. These results are consistent with those reported for *Lepidium sativum* L., *Amaranthus viridis* L., *Brassica carinata* A. Braun, *Apocynum pictum* Schrenk, and *Glycine max* (L.) Merr. (soybean) (Li *et al.* 2009; Jiang *et al.* 2018; Gayathri *et al.* 2022; Shakoor *et al.* 2023; Iannilli *et al.* 2024).

The radicle is the first structure to come into contact with the surrounding environment, whose properties can promote, limit, or even halt the development of the seedling by affecting young radicle tissues, especially the meristems, which are highly sensitive to various stress conditions. Although the nature and toxicity of Li are still largely unknown, previous results have shown that higher concentrations of Li strongly inhibit seedling growth. This study showed that Li concentrations from 25 to 100 mg L^{-1} have stimulatory effects on the development of the primary root in *B. oleracea* var. *capitata*, due to the specific, but still unknown, role of Li in plant metabolism. In contrast, Li concentrations of 200 mg L^{-1} or higher caused a delay in radicle elongation and root hair development, ultimately inhibiting seedling growth and resulting in complete plant mortality within a few days of germination.

Despite the initial stimulatory effects of low Li doses on radicle growth, they caused dose-dependent negative effects on subsequent seedling growth, resulting in reductions in root and shoot length, dry biomass, and cotyledon area, as

previously reported in various plant species (Naranjo *et al*. 2003; Li *et al*. 2009; Hawrylak-Nowak *et al*. 2012; Kavanagh *et al*. 2018; Kuloğlu *et al*. 2022; Kapoor & Hasanuzzaman 2024). These effects were particularly pronounced at a Li concentration of 100 mg L^{-1} (Li_{100} plants), indicating that *B. oleracea* var. *capitata* is more sensitive to elevated Li than *Brassica carinata,* which showed no toxic effects up to 200 mg L^{-1} (Li *et al*. 2009). In addition to the effects on plant growth, there was also a delay in the development of the Li_{100} plants, as evidenced by the complete absence of the first leaf.

Elemental status of plants. The Brassicaceae family includes up to 93 well-documented metal-hyperaccumulating species (Reeves *et al*. 2018; Srivastava 2020). Numerous species within this family, including several from the genus *Brassica*, accumulate and tolerate high levels of various metals, such as Cd, Cu, Ni, Pb, and Zn. This metal tolerance is enabled by efficient metal immobilisation mechanisms, including the high metal cation exchange capacity of their cell walls and the presence of various chelating agents which assist in metal transport and final sequestration within the vacuole. The observed dose-dependent accumulation of Li in the cabbage seedlings indicates that *B. oleracea* var. *capitata* strongly accumulates Li, as previously demonstrated for several other metals (Cd, Cu, Pb, Se, and Zn) (Bañuelos *et al*. 1996, 1997; Alkorta *et al*. 2004; Gisbert *et al*. 2006; Nouairi *et al*. 2006; Szczygłowska *et al*. 2011). Lithium was predominantly accumulated in the cotyledons compared to the roots, with concentrations reaching as high as 9060 mg kg^{-1} dw in the Li_{50} plants. Such high Li accumulation efficiency has previously been reported for the shoots of sunflower (*Helianthus annuus* L., 3292 mg kg^{-1} dw), lettuce (*Lactuca sativa* L.*,* 1607 and 1544 mg kg^{-1} dw), maize (*Zea mays* L., 695 mg kg^{-1} dw), and *Salvinia natans* L. (664 mg kg^{-1} dw) (Hawrylak-Nowak *et al*. 2012; Kalinowska *et al*. 2013; Török *et al*. 2022), when supplemented with the same Li salt and exposed to 50 mg Li L^{-1} in the nutrient solution. Similarly, effective accumulation in the leaves, exceeding 1000 mg kg^{-1} dry weight, has also been observed in *Beta vulgaris* L. (beet)*, Lactuca sativa*, *Brassica nigra* (L.) W. D. J. Koch (black mustard), and *Lolium perenne* L. cultivated in soil with only 5 mg Li kg^{-1}, with a BCF greater than 20 (Robinson *et al*. 2018). In *B. oleracea* var. *capitata*, the BCFs were very high, reaching 123 (Li_{25}), 181 (Li_{50}), and 69 (Li_{100}). The observed Li concentrations in different plant parts, along with its distribution pattern and predominant accumulation in the cotyledons, suggest that this alkali metal is effectively absorbed and readily translocated from the roots to the shoots. Although its specific transport mechanisms have not been identified, its concentrations and those of other examined cations suggest that Li likely uses the same transport mechanisms in the xylem and across cellular membranes as some other essential cations (Na^{+}, Ca^{2+}, Mg^{2+}, Cu^{2+}), as previously proposed by some authors (Kabata-Pendias & Mukherjee 2007; Shahzad *et al*. 2016). In addition, the highest concentrations detected in the cotyledons indicate its efficient upward translocation in the xylem via the transpiration stream, as reported by Kastori *et al*. (2022).

Literature data reveal different patterns of Li accumulation in monocotyledonous and dicotyledonous plants. In some species, it is concentrated in the roots, while in others, such as maize and sunflower, it is predominantly accumulated in the leaves (Hawrylak-Nowak *et al*. 2012; Antonkiewicz *et al*. 2017). Although these contradictory results suggest that Li concentration and its distribution within the plant depend on the plant species and the physiological mechanisms involved in metal accumulation and tolerance, variations in experimental conditions such as the type of Li salt used, growth medium (hydroponic solution or soil), pH, and duration of the experiment also play a significant role (Schrauzer 2002; Kalinowska *et al*. 2013; Franzaring *et al*. 2016). Thus, previous experimental data indicate that Li is more available and more efficiently absorbed by plants cultivated in hydroponic media compared to soil.

The strong synergistic effects of applied Li on the K, Ca, Cu, Mn, and Zn concentrations detected in the *B. oleracea* var. *capitata* roots could be related to the effect of elevated chlorides rather than increased Li. Given that one of the properties of chloride ions is their high stability in the soil solution, it could be suggested that the synergistic effect is based on the increased activity of cations in the nutrient solution due to the high concentration of soluble anions (Jakobsen 1992). Such an effect of chloride ions on cation absorption has been reported in *Phaseolus vulgaris* L., *Hordeum vulgare* L., *Zea mays*, and *Bassia* sp. (Jakobsen 1992; Curtin & Wen 2004). Recent investigations on rice plants cultivated in hydroponic medium showed that Cl^- increased the absorption of Cd by the root system, subsequently leading to high Cd concentrations in brown rice due to the over-expression of *OsNRAMP5*, *OsNRAMP1*, and *OsIRT1* transporter genes (Guo *et al*. 2024). Since these transporters are involved in the transport of one or more metals in plants, such as Mn, Zn, Cd, and Fe (Lee & An 2009; Takahashi *et al*. 2011; Ishimaru *et al*. 2012a, b; Yang *et al*. 2014; Chang *et al*. 2020), it could be assumed that their over-expression could also have led to increased Mn and Zn accumulation in both the roots and cotyledons of the investigated cabbage plants. In contrast to the pattern observed in the roots, the predominant Li accumulation in the cotyledons of the *B. oleracea* var. *capitata* plants significantly reduced the contents of Na, Ca, Mg and Cu in the Li_{100} plants. The Li-induced alterations in the nutrient balance resulted in changes to the stoichiometric ratios among all the investigated cations, which could potentially impact metal homeostasis within the plant cells and disrupt the functioning of metal-dependent processes.

While specific transport mechanisms for Li have not been documented, numerous studies indicate that Li competes for transport mechanisms and binding sites typically reserved for closely regulated cations. Increasing Li concentration results in a decrease in Na in the stems and cotyledons, indicating that Li largely uses Na channels for entry into cells, as shown in previous studies on animal cells and tissues (Richelson 1977; Thomsen & Shirley 2006; Uwai *et al*. 2014). Decreasing Na in parallel with increasing Li concentrations in all plant parts leads to markedly decreased molar ratios. A previous detailed study of the structure and function of voltage-gated sodium channels found that Li and Na have roughly equal permeability (Naylor *et al*. 2016). It can be assumed that the detected antagonistic effects of Li are probably determined by the rates at which Li and Na ions enter the pore, and when lithium ions are dominant in concentration, they are preferentially passed through channels over sodium ions.

The strong antagonistic effects of Li on Ca and Mg accumulation in the stems and cotyledons may be related to the similar sizes of their bare and hydrated ions. The ionic radius of Li is 0.60 Å, while that of Mg is very close at 0.72 Å; the radius of the hydrated Li ion is 3.82 Å, whereas those of the hydrated Mg and Ca ions are 4.28 Å and 4.12 Å, respectively (Zhong *et al*. 2015). Due to their physical similarities, lithium may interact with one of two distinct ionic sites at the catalytic units of proteins, to which Mg^{2+} and Ca^{2+} normally bind, thus altering their charges, structure, and activities (Mørk & Geisler 1987; Geisler & Mørk 1990). It was also found that lithium displaces Mg^{2+} and Ca^{2+} from their binding sites on membranes due to its small radius and high polarisation strength (Fossel *et al*. 1985; Vanyo *et al*. 1991). Additionally, Li^+ can exchange with Ca^{2+} in membrane-bound Ca^{2+}/cation antiporters causing its extrusion from the cell, thus playing a key role in disrupting Ca^{2+} homeostasis and various Ca-dependent processes within the cell (Refaeli *et al*. 2016). One such process is Ca-signalling, based on the role of Ca^{2+} as a second messenger, whose influx via Ca-channels, efflux via Ca-transporters, and binding to various Ca-binding proteins is highly regulated and results in appropriate cellular responses to stimuli, such as changes in mineral element content or elevated heavy metal concentrations (Wang *et al*. 2023). Investigations by Briggs *et al*. (2016) detected the

broad biological mode of action of Li, realised through the formation of a bimetallic (Mg-Li)ATP complex, thus revealing lithium's bioactive form through its association with phosphate-containing ligands or cofactors of receptors and enzymes, modulating their activities. Among the direct consequences of Mg substitution by elevated Li is the disruption of normal chlorophyll formation and a decline in chlorophyll content, as shown in this and numerous previous studies (Li *et al*. 2009; Hawrylak-Nowak *et al*. 2012; Török 2022). Owing to this inhibitory effect of Li on the functionality of various biomolecules containing one of these two metals, it inevitably interferes with different Mg- and Ca-dependent processes, as previously confirmed in various microorganisms, plants, and animals (Mørk & Geisler 1989; de Roos *et al*. 2001; Naylor *et al*. 2016; Shahzad *et al*. 2016).

The literature includes various and conflicting data regarding the effects of Li on K content in plants, ranging from clearly competitive interactions, including K leaching from the leaves, to synergistic effects (Magalhães *et al*. 1990; Naranjo *et al*. 2003; Makus *et al*. 2006; Hawrylak-Novak 2012). Our analysis did not show any significant changes in the K content in the aboveground plant parts. Despite strong Li accumulation in the cotyledons, the stable potassium content indicates a highly selective K-channel mechanism on both the extracellular and intracellular sides of the channel, enabling normal K influx into the cell and preventing its leaching in the presence of high Li. The current understanding of the selectivity mechanism in potassium channels from the extracellular side is based on the presence of K-selective binding sites, which do not bind the smaller Li ions (Morais-Cabral *et al*. 2001; Zhou *et al*. 2001; Lockless *et al*. 2007). Investigations of potassium-channel selectivity at the intracellular side in *Streptomyces lividans* have shown that K-channels distinguish between K, Na, and Li ions, and prevent Na^+ and Li^+ from permeating through the channel in the presence of K^+ (Thompson *et al*. 2009).

While the data on the concentrations of the studied elements clearly illustrate the pattern of their changes and can indicate antagonistic or synergistic relationships, their molar ratios are far more objective and, depending on the elements' atomic weights, can differ considerably from their weight ratios. This is particularly evident when comparing the concentrations of two elements with significantly different atomic weights, such as Li, the lightest metal, and Ca, which has an atomic weight almost six times higher. The stoichiometry of the elements therefore provides a clear indication of their molar ratios, potential interactions, and their consequences for the plant organism.

Effects of elevated lithium on antioxidant enzymes activities. Metal-induced excessive formation of reactive oxygen species (ROS) is the primary trigger for the activation of antioxidant enzymes in plants (Shahid *et al*. 2014; Natasha *et al*. 2019). Significantly elevated activity of both SOD and class III peroxidase, detected only in plants treated with the highest Li concentration (100 mg L^{-1}), suggests a Li-induced increase in superoxide anion radical and consequently H_2O_2 production. Class III peroxidases are typically localized in the vacuole and plasma membrane and are secreted into the cell wall where, in the presence of phenolic compounds as electron donors, they remove H_2O_2 and eventually produce phenolic polymers (Kidwai *et al*. 2020). The phenolic polymers formed by the activity of class III peroxidases in the cell wall serve to strengthen the cell wall and limit the entry of metals into the cell. It can be hypothesised that as with many other metals in excess, the cell wall may play a role in Li immobilisation and function as a crucial defensive barrier against Li-induced stress. This is consistent with the findings of Shakoor *et al*. (2023) who reported that Li in soybean was primarily deposited in the cell wall and vacuole. However, the observed increase in POD activity should be interpreted only as a relative change in the total POD activity in the cell. While CAT activity in the roots remained

largely unchanged, its activity in the leaves doubled, suggesting that the level of accumulated Li contributes to the excessive formation of H_2O_2. In contrast to the observed changes in CAT activity, APX activity in the roots decreased significantly in all the Li-treated plants, showing a dose-dependent decrease, while in the leaves it remained similar across all the treatments. Such a decline in APX activity may not necessarily result from changes in APX content or function, but could be due to the inhibition of another component of the ascorbate–glutathione cycle as a reactive oxygen scavenging system. For instance, this could be a consequence of the depletion of reduced glutathione (GSH), which is directly involved in redox homeostasis, as a substrate for GSH peroxidases (GPX), or in the production of phytochelatin (Kapoor & Hasanuzzaman 2024). Although there are no studies on the relationship between glutathione and Li in plants, numerous studies performed on animal tissues and cells have shown that elevated Li content depletes the GSH pool in the cell due to its oxidation by GPX (Malhotra & Dhawan 2008; Khan *et al*. 2010; Ahmad *et al*. 2011; Eskandari *et al*. 2012; Ullah & Khan 2014).

CONCLUSION

Although the effects of elevated Li concentrations in plants remain largely uncharacterised, available data indicate its hormetic effects, with a stimulatory influence at low concentrations and various negative effects at high concentrations. Increased Li content induces specific changes in the absorption and final concentrations of the analysed cations in the plant, resulting in the altered stoichiometric coupling of several elements. This suggests that the plant's internal balance between different nutrients can be seriously disturbed by a Li-contaminated environment, leading to a mismatch between the nutrient requirements of plants for growth, development, survival, and reproduction. To thoroughly understand the complex and long-term effects of elevated Li on the plant organism, comprehensive studies of plant physiology, growth, and development throughout the plant's life cycle or until maturity are needed. Among the various methodologies and tests, these may include investigations of pollen production and germination levels, the detection of the number, weight, and germination of the seeds produced, the evaluation of seedling viability, and analyses of genotoxic effects, and levels of gene expression. This would allow for the assessment of the potential long-term effects of elevated Li on natural plant populations, plant communities, and ultimately biocenoses in Li-contaminated areas.

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COMPREHENSIVE PLANT BIOLOGY

REZIME

Uticaj litijum hlorida na klijanje semena, rast i antioksidativni odgovor biljaka *Brassica oleracea* var. *capitata*

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Široka upotreba litijuma (Li) u industriji, nizak stepen reciklaže proizvoda sa njegovim visokim sadržajem, rudarstvo i prerada litijumom bogatih ruda doveli su do toga da ovaj element u novije vreme dobije status zagađivača životne sredine sa negativnim biološkim i ekološkim efektima. Uprkos brojnim istraživanjima tokom poslednje dve decenije, efekti Li na biljke i dalje su u velikoj meri nepoznati i često kontradiktorni. U ovom radu dat je prikaz efekata povišenih koncentracija Li, dodatog u tečni medijum u obliku LiCl, na *Brassica oleracea* var. *capitata* (kupus). Ispitivan je uticaj na klijanje semena, a zatim i na rast biljaka, njihov mineralni status, sadržaj fotosintetskih pigmenata, i aktivnost antioksidativnih enzima nakon dve nedelje gajenja. Stopa klijanja semena ostala je visoka (>80%) u svim tretmanima. U ranim fazama razvoja biljaka, najniže primenjene koncentracije Li (25–100 mg L^{-1}) stimulisale su izduživanje korena i razvoj korenskih dlačica, dok su više koncentracije dovele do smanjenog rasta i čak prouzrokovale prevremenu smrt klijanaca, što ukazuje na hormezis. Tokom naredne dve nedelje gajenja, litijumom tretirane biljke ispoljile su dozno-zavisno smanjenje rasta i postignute biomase. Litijum, koji se ponajviše akumulirao u listovima, uticao je na akumulaciju i translokaciju više ispitivanih mineralnih elemenata u biljci, remeteći njihov sadržaj i molarne odnose. Povećani sadržaj Li u listovima je rezultirao smanjenim sadržajem fotosintetskih pigmenata. Takođe je došlo i do značajnih promena u aktivnostima enzima antioksidativne zaštite: superoksid dismutaze (SOD), katalaze i peroksidaza klase III (POD) u listovima, kao i SOD, askorbat peroksidaze i POD u korenu, što ukazuje na povećano stvaranje reaktivnih kiseoničnih vrsta. Sveukupni rezultati pokazuju da i pored početnog stimulatornog efekta, čak i niske doze Li u narednom periodu ispoljavaju inhibitoran efekat na rastenje i ispitivane fiziološke parametre biljke. Stoga su neophodna znatno dugotrajnija ispitivanja koja bi obuhvatila različite parametre rastenja, razvića i funkcionisanja biljke do njenog sazrevanja. Ovo je posebno važno imajući u vidu da je kupus biljka od ekonomskog značaja i među onima koje se najčešće koriste u ishrani ljudi na teritoriji Srbije i Evrope.

Ključne reči: kupus, enzimi, antagonizam metala, tolerancija metala, oksidativni stres